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<b>(54) Title:</b> A HYPOALLERGENIC COMPOSITION CONTAINING TOLEROGENIC PEPTIDES INDUCING ORAL TOLERANCE		
<b>(57) Abstract</b>  A hypoallergenic composition for the induction of protein tolerance in at risk individuals of protein allergy, consisting of (i) a "non allergenic" protein extensively hydrolysed basis and/or of (ii) a free amino acid basis, said composition comprising as the active ingredient at least one tolerogenic peptide of the allergenic protein.		

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## **A hypoallergenic composition containing tolerogenic peptides inducing oral tolerance**

### **Field of the invention**

5 This invention relates to a hypoallergenic composition containing specific tolerogenic peptides of proteins, said composition can induce oral tolerance to the native proteins. It also relates to the use of tolerogenic peptides of milk protein for the preparation of a composition inducing immunological tolerance to milk proteins.

### **Background of the invention**

10 During the common process of nutrition, dietary proteins are presented to the immune system via the gut, followed by an immune unresponsiveness to the ingested nutrients. This vital phenomenon, called oral tolerance, is efficient for the large majority of people. If oral tolerance fails, food allergy occurs, requiring the strict avoidance of the incriminated food.

15 Targeted food avoidance represents a hard task for adult patients with food allergy. For example, to strictly remove cow's milk from the diet of allergic infants might be even more difficult, especially if breastfeeding is not possible or desired.

20 The allergies to cow's milk and to the formulas containing cow's milk adapted to the needs of infants are due to the fact that the proteins of cow's milk differ from the proteins of mother's milk and can constitute allergens. Among whey proteins,  $\beta$ -lactoglobulin is the major component and is a strong allergen.

25 Besides breastfeeding, the primary recommendation for prevention, hypoallergenic formulae are systematically prescribed to "at risk" newborns, namely asymptomatic infants with atopic parents.

30 In contrast to adapted formulae, cow's milk proteins have been hydrolyzed in hypoallergenic formulae, to decrease the potential allergenicity. This approach has been demonstrated to be efficient in order to prevent sensitization by native proteins present in the adapted formulae.

35 Thus, in US 4,293,571 a protein hydrolysate was prepared by pancreatic hydrolysis, coagulation of non-hydrolysed proteins by a heat treatment and ultrafiltration to eliminate the coagulated residual proteins and the macropeptides

which could constitute allergens. Also, US 5,039,532 provides an improved process for the preparation of a hydrolysate of animal milk proteins substantially reduced in allergens, in which a whey product was subjected to enzymatic hydrolysis.

5 Two different types of hypoallergenic formulae are proposed to high-risk babies: partially and extensively hydrolyzed formulae, differentiated by the extent of hydrolysis of the native proteins.

But as is clear from the art cited above, the primary focus to date in dealing with allergies to cow's milk has been to find preparations which will not induce an allergic response, i.e. to provide non-allergenic formulations.

10 Nonetheless, while such formulations have permitted a person allergic to cow's milk to avoid an allergic response, they do not solve the problem which is to enable a person to drink unaltered milk products.

15 More recently, it has been investigated that partially hydrolysed milk formulations are not only of reduced allergenicity but can induce immunological tolerance to milk proteins. Extensively hydrolysed formulae are specially designed for treating patients allergic to cow's milk proteins but their ability to induced long-term oral tolerance is questioned.

20 Thus, EP 0 629 350 discloses the use of non-allergenic whey protein hydrolysates which are said to be capable of inducing cow's milk protein tolerance. Although this patent application indicates that whey protein hydrolysates substantially free of allergenic proteins could be used to induce cow's milk protein tolerance in children at risk of cow's milk allergy, the present inventors found on analysing other non-allergenic whey protein hydrolysates that non-allergenicity did not necessarily translate into the ability to induce cow's milk protein tolerance. Indeed, some of the formulations exhibiting the highest degree of non-allergenicity were found to be unsuitable for inducing cow's milk protein tolerance.

30 Thus, it is clear that the art lacked the information to provide formulae that would be the most suitable for tolerance induction.

### Summary of the invention

35 This invention provides a hypoallergenic composition for the induction of protein tolerance in at risk individuals of protein allergy containing (i) a "non-

allergenic" extensively hydrolysed proteins basis and/or (ii) a free amino acid basis, said composition comprising as the active ingredient at least one tolerogenic peptide of the allergenic protein.

5 In a preferred embodiment, said tolerogenic peptides are present in the form of (i) isolated tolerogenic peptidic fractions of hydrolysis of proteinaceous material containing the allergenic protein and/or (ii) synthetically prepared tolerogenic peptides.

10 This composition contains a source of nitrogen which may provide 7 to 25% of the total energy, a source of carbohydrates which may provide at least 28 to 66% of the total energy, a source of lipids which may provide at least 25 to 60% of the total energy and at least one tolerogenic peptide of the different proteins.

15 A major advantage of this composition is to induce oral tolerance in "at risk" individuals, in order to avoid eventual sensitization by use of native tolerogens. Moreover, the tolerogenic peptides derived from protein hydrolysis offer both hypoallergenic and tolerogenic properties and induce oral tolerance at the humoral and cellular levels.

20 This composition is particularly intended for individuals at risk of milk protein allergy.

25 Another aspect of the present invention is the use of tolerogenic peptides of milk proteins for the preparation of a hypoallergenic composition intended for mammals susceptible to cow's milk allergy.

30 In a preferred embodiment tolerogenic peptides are from milk origin and particularly from  $\beta$ -Lactoglobulin ( $\beta$ -LG),  $\alpha$ -lactalbumin, bovin serum albumin or casein origin.

35 For the preparation of said composition, tolerogenic peptides may be used in the form of peptidic fraction containing the following peptides :  $H_2N$ -I-D-A-L-N-E-N-K-COOH,  $H_2N$ -V-L-V-L-D-T-D-Y-K,-K-COOH or  $H_2N$ -T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH from  $\beta$ -Lactoglobulin.

In another aspect, the invention provides a method for the preparation of tolerogenic peptides useful in the induction of protein tolerance in at risk individuals to protein allergy, wherein:

- 5 (i) a proteinaceous material containing the allergenic protein is hydrolysed to a degree of hydrolysis of about 10 to 50 %;
- (ii) then treated to inactivate residual enzyme activity;
- (iii) the protein hydrolysate solution is clarified and submitted to precipitation treatment or passed into a chromatography column filled with appropriated resin to extract tolerogenic peptidic fractions.

10

### Detailed description of the invention

15 In the present invention, the term tolerance is to be understood as a state of specific immunological unresponsivness. Both humoral (antibodies) and cell-mediated (lymphocyte ....) pathways of the immune response may be suppressed by tolerance induction. A breakdown of oral tolerance is considered to be the underlying cause of food allergy.

20 The term allergen is to be understood as a protein or macropeptide capable of initiating allergic reactions in humans, particularly at risk infants or nurslings. Infants are considered being "at risk" of protein allergy when either one, two parents or one sibling is atopic.

25 The term tolerogenic peptides is to be understood as proteic fragments, corresponding to parts of the native protein, sized from 200 to 6000 Da (3 to 50 amino acids), and preferably between 500 to 3000 Da and being able to induce specific oral tolerance to native proteins.

30 The term "non-allergenic" basis is to be understood as a nitrogen source containing a well-balanced amino-acids composition. The "non-allergenicity" is defined for milk proteins as residual allergenicity of individual whey proteins not exceeding 1 ppm and as residual allergenicity of total caseins not exceeding 10 ppm.

35 The hypoallergenic composition may contain as a source of nitrogens, peptides or free amino acids and particularly from milk proteins such as whey

proteins,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, bovine serum albumin, casein acid, caseinates, or  $\alpha$ ,  $\beta$ ,  $\kappa$ -casein, for example. The source of nitrogen can provide at least 7 to 25 % of the total energy.

5 As a source of carbohydrates, lactose, saccharose, starch or maltodextrin may be used. Carbohydrates may provide at least 28 to 66 % of the total energy.

Vegetable oils or butter oil are preferably used as a source of lipids which may provide at least 25 to 60 % of the total energy.

10

Vitamins, oligoelements and minerals can be added in an amount sufficient to meet daily requirements.

15

The composition according to the present invention comprises as the active ingredient at least one tolerogenic peptide of the allergenic protein, said tolerogenic peptide has been selected for its ability to induce oral tolerance.

20

The tolerogenic peptides can be obtained by enzymatic hydrolysis of proteinaceous material containing the allergenic proteins that are responsible for allergies in at risk individuals, followed by isolation of tolerogenic peptidic fractions. These peptidic fractions enriched in said tolerogenic peptides can be obtained by separation of the protein hydrolysate. The tolerogenic peptides may also be present in the composition in the form of synthetically prepared tolerogenic peptides.

25

The composition contain an amount sufficient to induce oral tolerance which is preferably the one which allows a complete oral tolerance induction, namely the one which prevents from any reaction after DBPCFC (double blind placebo controlled food challenge) performed with cow's milk. Accordingly, tolerogenic peptides may be present in an amount of about 0.01% to 10% (nitrogen source of the protein), for example and preferably about 0.1 to 0.2 % of total peptides.

30

In the particular case of tolerance to milk proteins, the composition may contain tolerogenic peptides from milk origin such as  $\beta$ -lactoglobulin or caseins, for example. Tolerogenic peptides may thus be in the form of a peptidic fraction comprising at least one of the following peptides :  $\text{H}_2\text{N-I-D-A-L-N-E-N-K-COOH}$ ,

35

H<sub>2</sub>N-V-L-V-L-D-T-D-Y-K,-K-COOH or H<sub>2</sub>N-T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH from  $\beta$ -lactoglobulin.

5 In a preferred embodiment, a method for the preparation of tolerogenic peptides comprises the following steps:

- (i) a proteinaceous material containing the allergenic protein is hydrolysed to a degree of hydrolysis of about 10 to 50 %;
- (ii) then treated to inactivate residual enzyme activity;
- 10 (iii) the protein hydrolysate solution is clarified and submitted to precipitation treatment or passed into a chromatography column filled with appropriated resin and tolerogenic peptidic fractions are recovered.

This preferred method is well suited to treatment of hydrolysates prepared from various protein concentration ( $N_{tot} \% = N * 6.38$ ) for modifying the ratio of  
15 tolerogenic activity by residual antigenicity from proteinaceous material. If one defines arbitrarily the antigenicity of a native protein to be  $10^6$  (as  $10^6$   $\mu\text{g/g}$  of protein), and the tolerogenic response to be 1, then, for a native protein, this ratio is  $10^{-6}$ . Therefore, the ratio qualifying the tolerogenic activity of one given fraction or tolerogenic peptide should be at least  $2 \times 10^{-2}$ .

20 The proteinaceous material to be treated may be any composition containing protein material and in particular solution or dispersion of milk proteins : whey proteins, acid whey protein, sweet whey proteins, whey protein concentrates, whey protein isolate, demineralized whey powder or caseinates, for example.

25 In general, the protein content may vary within the range of about 70 to 95% by weight but the starting material is preferably as rich in protein as possible.

30 The proteins present in the proteinaceous material can be modified with proteolytic enzymes into protein hydrolysate having a degree of hydrolysis ( $\alpha$ -amino-N/ $N_{tot}$ ) of preferably about 10-50 %.

35 The proteolytic enzymes may be for example, from animal or vegetable origins (pepsin, chymotrypsin, trypsin, intestinal mucosa extract, pancreatic extracts, chymosin, papain, bromelain, ficin), bacterial or fungi origins (serine and metalloproteases from *Bacillus subtilis*, *Bacillus licheniformis*, *Aspergillus oryzae*,



*Aspegillus wentii* and acidic proteases from *Aspergillus orizae*, *Aspergillus wentii*, *Mucor miehei*, *Mucor pusillus*, *Endothia parasitica*) or a combination of these.

5 During hydrolysis, concentration of proteinaceous material in solution or in suspension is preferably around 5-20% by weight and could be pasteurised before introducing proteases. The ratio enzyme/protein may be 0.1-10% weight/weight and preferably of about 0.25 to 4%.

10 Hydrolysis may be conducted at a temperature of about 35°C to 65°C, during 30 minutes to 10 hours, preferably 30 min to 4 hours at pH values within the range 2.5 to 11, preferably 4.5, 7.0, 8.0, and 8.5. If desired the pH of the solution can be adjusted and regulated with citric acid, food grade HCl or NaOH, NH<sub>4</sub>OH, KOH, Ca (OH)<sub>2</sub> for instance at a concentration of 2N pure or in blend.

15 Then, the protein hydrolysate may be submitted to a heat treatment of about 0.1 to 10 min at a temperature of about 70 to 110°C to inactivate residual enzymes (i.e. proteases).

20 The protein hydrolysate solution thus obtained can be clarified by centrifugation and/or ultrafiltration to remove insoluble and intact proteins respectively, and the clear solution is recovered. It is possible to use at industrial scale different type of membranes (spiral, tubular, flat, allow fibbers) made with different materials (minerals, polysulfone, ...) and having different cut off limits between 1.000 and 100.000 Daltons. Depending on the type of enzyme, the  
25 hydrolysis conditions and the type of membranes the modification of the tolerogenic fractions might be sufficient at this step.

The recovered clear hydrolysate solution can, if desired, be concentrated by evaporation to a dry solid content of 10-50% for a subsequent treatment or spray  
30 dried if enrichment in tolerogenic peptides is sufficient.

The protein hydrolysate solution thus obtained can be submitted to precipitation treatment by solvent, acid, or salts, for example, followed by a centrifugation. In the precipitation treatment, concentration of hydrolysate solution  
35 increases the yield and reduces the quantities of solvent. For example, ethanol may be added to obtain a final concentration within 15-60% volume/volume at a

temperature of about 4°C to 25°C. After one hour of incubation, a centrifugation (30 min at 4500 g) may allow to separate soluble and insoluble peptides. Depending on the proteolysate we can use acid (phosphoric or chlorhydric, for example) or phospho-calcic precipitation. Then, solvents can be removed by evaporation and salts by electrodialysis for instance.

The clear solution and the insoluble fraction are preferably recovered.

The protein hydrolysate solution thus obtained may be passed into a column filled with adsorption, ion exchange or hydrophobic resin at a flow rate of 0.1-4 column volumes per hour at a temperature of about 4°C to 60°C. Before the chromatography treatment, the protein hydrolysate can be concentrated to provide a solution having a dry solid content of 8-35% by weight.

During chromatography, a fraction of peptide is absorbed into the resin by passing the hydrolysate solution into a column filled with the convenient support at a rate of 0.1-4 column volumes per hour. It is possible to use at industrial scale the different types of chromatography as: ions exchange, hydrophobic interactions, reverse phases, adsorption (hydroxyapatite, active charcoal, polystyrene base hydrophobic resins...) or covalent chromatography, for example.

In the chromatography treatment, the amount of hydrolysate solution per litre of resin filled column can be as high as 5 litres with the respect to dry solids of 10%. Preferably, a hydrolysate solution having 20-1000g of dry solid per litre of resin is passed into the resin filled column. The chromatography treatment may be carried out at a pH of about 2 to 10 preferably 6-8, for the clarified hydrolysate solution. The chromatography treatment can be conducted at a temperature of about 4°C to 60°C.

For example, the chromatography treatment to select tolerogenic fractions from  $\beta$ -lactoglobulin may consist in using:

- a strong cationic resin equilibrated with 0.1 N HCl at a flow rate of 1 volume/hour. The non-retained fraction was eluted with 3 volumes of water, the second fraction (fraction containing tolerogenic peptides) was eluted with 0-0.5 N NaOH, and the third fraction was eluted with 0.1 N HCl.

- a reverse phase (C18) resin equilibrated with pure water. Non retained fraction was eluted with water, then step by step (20% and 40% of ethanol) the second and the third were recovered.
- a strong anionic resin equilibrated with 0.1N NaOH. Non retained fraction was eluted by 3 volumes of water. The second fraction was eluted with 0.5N HCl, the third one with 0.1 N NaOH.

The most preferred method is to treat with resin a neutral solution, in that case, no pH adjustment is required-after hydrolysis step and the salt content of the product will be lower .

To conclude the chromatography treatment, the column can be eluted with pure water, then water containing salts, buffer, acids, bases, or organic solvents at a temperature of 4-60°C. Elution is realised step by step or by a gradient of concentration. The solutions that have passed through the column are recovered. If necessary, salts, solvents, acids, bases, are removed from the recovered solution, and the recovered solutions can be concentrated to a dry solids content of 35-65% and spray dried.

These peptides are then specific fragments corresponding to a part of the native protein sequence or to a part of the specific tryptic peptides of hydrolysed protein.

These tolerogenic peptides can be used for the preparation of a composition inducing oral tolerance to native proteins, said composition is intended for mammals susceptible to protein allergy and particularly human and pets.

The following examples are given by way of illustration only and in no way should be construed as limiting the subject matter of the present application.

## Figures

Figure 1 represents specific oral tolerance induction in mice fed once with either TTH  $\beta$ -LG, F2 or F(7+9). A: Humoral (dark bars) and mucosal (gray bars) anti- $\beta$ -LG IgE response. B:  $\beta$ -LG-specific DTH response (•) are individual foot pad increments and (-) are means from 10 mice per group. C: Specific ( $\beta$ -LG) and non

specific (PHA) proliferative responses of splenic cells. Splenic lymphocytes from mice fed with either saline (◆), TTH  $\beta$ -LG (■), F2 (▲), or F (7+9) (●) were isolated and subsequently stimulated with decreasing concentrations of antigens.  $^3\text{H}$  thymidine incorporation was measured after 120 hours of culture.  $(^3\text{H})\text{Tdr}$  incorporation results were expressed in cpm, as a mean of triplicate cultures, the blank-subtracted mean was then plotted respectively against  $\beta$ -LG or PHA concentration.

Figure 2 represents primary sequences assigned to tryptic peptides identified in  $\beta$ -Lactoglobulin hydrolyzate. Vertical bar represents disulfide bond.

### Examples

#### Example 1: Selection and characterization of the tolerogenic peptides of $\beta$ -Lactoglobulin

In the first following exemple, experiments were performed to select the tolerogenic peptides of  $\beta$ -LG.

$\beta$ -LG was digested by TPCK treated trypsin in conditions selected to provoke its mild digestion. The size and the molecular weight distribution of the resulting peptides from our  $\beta$ -LG hydrolysis model, respectively ranged from two to twenty-three aminoacids, and from 247 to 2719 Da. The tolerogenic properties of the resulting peptides have been assayed in an oral tolerance experimental mouse model

### Methods

#### a) Animals

Female Balb/c mice were obtained from IFFA-Cr do (L'Abresle, France). They were all bred and raised on a milk free diet. The mice were 3 weeks old at the start of the experiments.

#### b) Preparation of tolerogenic fractions

In order to be digested,  $\beta$ -LG (220 grams) was dissolved in bi-distilled water at 5% (w/w) final concentration. The  $\beta$ -LG was digested by TPCK treated trypsin, using an Enzyme/Substrate (E/S) ratio of 1/100(w/w) at 40°C, pH 7.6 under constant stirring. After one hour hydrolysis, same amount of enzyme was added to give a  
5 2/100 (w/w) final E/S ratio. After 4 hours hydrolysis, the reaction was stopped by inactivation of trypsin at 85°C for 5min. Total tryptic hydrolysate of  $\beta$ -lactoglobulin (TTH  $\beta$ -LG) was then lyophilized. The digested  $\beta$ -LG products were separated by preparative chromatography on a cationic resin.

10 15 different fractions were obtained, individually nanofiltered to concentrate and to eliminate salts, diafiltered, dialyzed, lyophilized and dry stored at room temperature until *in vivo* experiments.

Each fraction was further characterized by its peptide content using reverse  
15 phase High Performance Liquid Chromatography. Considering TTH  $\beta$ -LG as reference, enrichment and impoverishment of peptides in each fraction was appreciated by area detected at 214 nm using iso nitrogen injections.

#### c) Oral tolerance induction and immunization procedure

20

Gavages were orally administered to the mice at age 22 days, by gastric feeding of native  $\beta$ -LG (5 mg/g of body weight), various amounts of TTH  $\beta$ -LG, or various amounts of the different  $\beta$ -LG peptidic fractions. Control mice were fed saline water. 5 days later, all mice were immunized  $\beta$ -LG and OVA (Ovalbumin,  
25 gradeV, Sigma), as a non-related antigen for testing the specificity of the immune response, 21 days after systemic challenge, a Delayed Type Hypersensitivity (DTH) evaluation was done by duplicate thickness measures of the left, rear footpad prior to, and following, immunization with  $\beta$ -LG.

#### 30 d) Evaluation of the immune response

24 hours after DTH immunization, individual increases in foot pad thickness of those injected the day before were measured. Differences between these two measurements were expressed in  $\Delta$  thickness (millimeters) and used in group  
35 comparisons. Then, from all mice, blood samples were obtained, spleens were taken and pooled according to group of treatment. Splenocyte specific proliferation assays

were performed for each group. Intestinal contents were individually collected. Serum and intestinal samples were rapidly frozen at -80°C, until assays. Specific IgE against  $\beta$ -LG and against OVA were determined both in seric and intestinal samples.

5 **e) Specific IgE antibody assays**

Serum and intestinal fluid dilutions were assayed in duplicate for anti- $\beta$ -LG and anti-OVA IgE antibodies by ELISA. Pooled samples from twenty non-immunized female mice were used as negative controls in each plates. Titers were  
10 determined by calculating the dilution of the sample which gave twice the absorbance of the negative control. Titers were expressed as the  $\log_{10}$  of the reciprocal of the dilution.

15 **f) Cell cultures**

Spleen cell solutions were homogenized and purified. Cells were cocultured in the presence of  $\beta$ -LG or of phytohaemagglutinin A. ( $^3\text{H}$ )Tdr (Amersham, Zurich) was added in the final 6 hours of culture and the plates were harvested and analyzed by scintillation counting. Stimulation indices were calculated as the ratio of blank-subtracted test and control values expressed as the mean cpm ( $^3\text{H}$ )Tdr incorporation  
20 by triplicate cultures.

**g) Determination of  $\beta$ -LG IgG binding capacity**

25 An ELISA inhibition was used to determine  $\beta$ -LG epitopes in  $\beta$ -LG hydrolysate and in  $\beta$ -LG fractions.  $\beta$ -lactoglobulin titers were calculated from a 5-fold dilution  $\beta$ -LG standard curve run on each plate.

30 DTH responses, seric and intestinal IgE responses were compared using ANOVA single factor tests.

**Results**

35 **a) Characterization of fractions obtained from TTH  $\beta$ -LG**

In order to isolate the tolerogenic peptides from the TTH  $\beta$ -LG, large amounts of  $\beta$ -LG tryptic fractions have been produced by preparative chromatography. From each of them, 15 fractions (F1-F15) were collected and respectively pooled. The assigned sequences of  $\beta$ -LG tryptic peptides have been previously determined (fig.2).

- No specific peptide enrichment could be detected in fractions F1, F5 and F15.
- 3 different peptides T6, T17, and T18 present in fraction F2 were enriched, but were not concentrated in any of the other fractions.
- Similarly, T7 was specifically enriched in fraction F6.

Apart from these peptides, concentrated in only one given fraction, some others were enriched in several fractions. This was the case for T21, for instance, clearly detected at high rates in fractions F7, F9, and F10 (Table 1).

Fractions	F1	F2	F3	F4	F5	F6	F7
Masses (g)	52	13.9	11	11.8	2.57	1.94	4.37
Significantly enriched peptides	-	T6 T17 T18	T23	T23	-	T7 T9	T20 T21 T24
F8	F9	F10	F11	F12	F13	F14	F15
13.1	5.01	6.53	1.05	3.46	1.9	2.67	28.8
T9 T12 T24	T12 T21	T10 T21	T11	T10	T10 T11	T10 T11	-

Table 1:  $\beta$ -LG fraction masses and significant peptide enrichment within fractions.

- b) Two different fractions of the  $\beta$ -LG tryptic hydrolysate presented tolerogenic properties:

Fractions were pooled according to their enriched peptides similarities and in proportion to their respective masses, and then tested *in vivo*.

- F1, F2, F5, and F15 were considered to be fed individually because they did not share peptide enrichment with the others fractions.
- F3 and F4 were pooled based on high concentration of T23;
- F8 and F9 were mixed to test the tolerogenic activity of T12;
- F7 and F9 were pooled according to enrichment of T20 and T21;
- F6, F11, F12, F13, and F14 were pooled together, each enriched in T9, T10, and T11.

Qualitative responses of the tolerogenic properties generated from feeding the different fraction groups at two different concentrations (0.5 or 0.125 mg/g of body weight) were evaluated. Induction or absence of induction of tolerogenic responses to  $\beta$ -LG has been determined by the statistical significance of the immune response.

Two of the eight tested fractions appeared to be active both in reducing seric anti- $\beta$ -LG IgE responses and  $\beta$ -LG specific splenocyte proliferation:

- F2, when given orally at 0.125 mg/g of body weight,
- F(7+9), at both tested doses.

#### c) Humoral, mucosal and cellular tolerance induced by oral administration of $\beta$ -LG tryptic fractions F2 and F(7+9)

According to our quantitative criteria, seric and intestinal anti- $\beta$ -LG IgE responses, DTH, and specific splenocyte proliferation, oral tolerance has been induced in mice fed either with F2 or with F(7+9) (Fig. 1).

For the two fraction fed groups, the seric anti- $\beta$ -LG IgE titers,  $3.58 \pm 0.18$  and  $3.21 \pm 0.29$  respectively for F2 and F(7+9), were not as low as when induced by TTH  $\beta$ -LG feeding ( $2.68 \pm 0.05$ ) (Fig 1A). However, specific levels of IgE were significantly lower than titers of control mice ( $4.16 \pm 0.05$ ) ( $p < 0.05$ ). Specific intestinal IgE was also decreased in mice fed TTH  $\beta$ -LG ( $1.66 \pm 0.32$ ) or fed the two  $\beta$ -LG tryptic fractions ( $1.1 \pm 0.34$  and  $1.9 \pm 0.21$  respectively for F2 and F(7+9)), assessing the orally induced down-regulation compared to the control group ( $2.32 \pm 0.64$ ). Fig. 1B shows that specific DTH was clearly reduced in mice fed with either TTH  $\beta$ -LG (0.074 mm) or each of the two tolerogenic fractions (0.063 mm and 0.062 mm respectively for F2 and F(7+9)) in comparison to local hypersensitivity measured in control mice fed only with saline (0.164 mm).



To confirm the achieved induction of oral tolerance on cellular immunity, specific and non-specific splenocyte proliferation assays were performed. Fig. 1C and D show that feeding mice either of the two elected  $\beta$ -LG fractions as well as TTH  $\beta$ -LG provoked a severe decrease of the proliferative response to  $\beta$ -LG in comparison to the one observed from splenocytes isolated from saline fed control mice. Indeed, stimulation indices were 0.225, 0.174, and 0.341, respectively for TTH  $\beta$ -LG, F2, and F(7+9) fed mice. As expected, the non-specific proliferative response due to PHA stimulation was not affected by the different feeding regimen.

#### d) Antigenicity of the $\beta$ -LG hydrolysate and $\beta$ -LG fractions

Measurement of residual  $\beta$ -LG epitopes performed for the different fractions by ELISA inhibition showed that amounts of  $\beta$ -LG epitopes present per g of proteins were highly different from one fraction to another, ranging from 15  $\mu$ g/g of protein for F1 up to 9240  $\mu$ g/g of protein for F13. These results also indicate that antigenic and tolerogenic sites might be distinctly located, suggesting that allergenicity and tolerogenicity could be uncoupled. Schematically, three different type of fractions can be described :

- a first type, represented by fraction F2, including basic peptides with a high tolerogenic potential associated to a very low antigenicity,
- at the other end, an F13 type fraction containing acidic peptides, highly antigenic and devoid of tolerogenic activity and, finally,
- in a median position, the peptides present in fractions F7 and F9 inducers of tolerance but still having notable antigenicity.

Fractions F2 and the mixture F(7+9) were both tolerogenic. Antigenicity of the tolerogenic fractions F2, F7 and F9 were lower than that of the total hydrolysate. Remarkably, the antigenicity of the tolerogenic F2 was found to be 53 times lower than the antigenicity of TTH  $\beta$ -LG. In these two tolerogenic fractions, the sizes of the potentially tolerogenic peptides were distributed between 8 amino acids for T6, and 23 amino acids for T21, given respective molecular weights between 915 and 2719 Da.

To be tolerogenic, peptides seem to require a precise balance between dose, size, sequence and structure. However, until now, correlation between peptide structure and tolerogenic properties has not been described in the literature.

**Example 2 : Obtention of tolerogenic peptides from whey protein**

5 In order to obtain tolerogenic peptides, 1 kg of whey protein isolate was dissolved in 7 litres of water at 50°C. The pH was adjusted at 7.5 with 2N KOH. The volume was adjusted to 14 litres with water at 50°C to obtain a 7% solution with respect of powder.

10 10 g of enzyme Trypsin+Chymotrypsin were added and the solution was allowed to hydrolysis during 120 min. The pH was maintained with an alkaline solution of 2N KOH and 2N NaOH. After the hydrolysis, the solution was heated to 80°C during 10 min. The solution was cooled to 40°C and ultrafiltered with 40,000 Da cut off membranes.

15 The clear permeate was treated by cationic resin conducted as followed in a final resin volume of about 180 ml. The resin was washed and equilibrated with 0.1N NaOH at a flow rate of 300 ml/h. 100 ml of clear permeate 40,000 Da were passed into the column. The non retained fraction was eluted by 540 ml of pure water. The second and the third were eluted by 900 ml of 0.5N HCl and 540 ml of 20 0.1N NaOH respectively.

The tolerogenic peptides are present in fraction F2 for example, in an amount sufficient to prevent from any reaction after DBPCFC performed with cow's milk.

25 **Example 3 : obtention of tolerogenic peptides from caseinates**

1 kg of natrium caseinate was dissolved in 7 litres of water at 50°C. The pH was adjusted at 7.5 with 2N KOH. The volume was adjusted to 10 litres with water at 50°C to obtain a 10% solution with respect of powder.

30 40 g of Alcalase enzyme were added and the solution was allowed to hydrolysis during 120 min. The pH was maintained with an alkaline solution of 2N KOH and 2N NaOH. Subsequent to the hydrolysis, the solution was heated to 80°C during 10 min. The solution was cooled to 40°C and ultrafiltered with 10,000 Da cut 35 off polyethylsulfone membranes.

Reverse phase chromatography: 300 ml of the clear permeate were passed into the column equilibrated at a flow rate of 3 ml / min. The non retained fraction was eluted with 300 ml of water, then the second fraction was eluted with 200 ml of ethanol gradient 0-20%. Finally the third fraction was eluted with 200 ml of ethanol gradient 20-40 %. The column was regenerated by 400 ml gradient ethanol 40-80 % v/v and equilibrated with 500 ml of pure water.

#### Example 4

The procedure was as described in example 3, except that the enzyme quantity and membrane cut off were changed by Alcalase 20 g and 40,000 Da respectively. The subsequent chromatography on cationic resin was conducted as followed : 150 ml of resin were washed 3 times by 500 ml of pure water then poured in a column which final resin volume was 180 ml. The resin was washed and equilibrated by 0.1N NaOH at a flow rate of 300 ml/h. 100 ml of clear permeate 40,000 Da were passed into the column at a flow rate of 300 ml/h. The non retained fraction was eluted by 540 ml of pure water. The second and the third ones were eluted by 900 ml of 0.5N HCl and 540 ml of 0.1N NaOH respectively.

#### Example 5

The procedure was as described in example 3, except that the reverse phase treatment was changed by a chromatography on a strong anionic resin as followed: 150 g of resin were washed 3 times by 500 ml of pure water, then poured in a column where the resin was washed and equilibrated with 0.1N HCl at a flow rate of 180 ml/h. 100 ml of clear ultra filtrate were passed into the column at a flow rate of 180 ml / h. The non retained fraction was eluted with 540 ml of water. The second and the third ones were eluted by 3 column volumes of 0.5N NaOH and 5 column volumes of 0.1N HCl respectively.

#### Example 6: Tolerogenic infant formula

The tolerogenic peptides T6 and T17 contained in tolerogenic fraction F2 were synthetically prepared. T13 contained in a non tolerogenic fraction was also synthesized as a negative control. Oral tolerance induction of these peptides were

tested in mice as described in example 1c) except that the dose is of 20 mcg/g of body weight.

### Results :

5

Peptides	tolerized anti- $\beta$ -LG IgE / control	Stimulation index (rate)	Stimulation index (GLM)
T17	2.67/3.39	0.305	0.405
T6	3.24/3.39	0.53	0.285
T6-T17(linked)	3.40/3.39	0.92	0.81
T13 (negative control)	3.20/3.39	1.7	0.87

The oral administration of T6 or T17 induces a cellular tolerance (specific inhibition of lymphocyte proliferation). T17 also induces a humoral tolerance by significantly decreasing specific anti-b-LG IgE.

10

The oral administration of T13 (negative control) do not induce any tolerance.

15

These two peptides T6 ( $\text{H}_2\text{N-I-D-A-L-N-E-N-K-COOH}$ ) and T17 ( $\text{H}_2\text{N-V-L-V-L-D-T-D-Y-K}_2\text{-K-COOH}$ ) have a high tolerogenic potential associated to a very low antigenicity. They can be used in food or pharmaceutical composition for inducing a protein tolerance in at risk individuals of protein allergy.

### Example 7: Tolerogenic infant formula

20

The composition for 100 g of powder contains 12.5 % of peptides (synthetized peptides T6 or T17 or tolerogenic peptides as prepared in example 2, represent about 0.1 to 0.2% of total peptides), 26 % of fats, 56 % carbohydrates (including lactose 39 %, maltodextrin 11 %, starch 6 %), traces of vitamins and oligoelements to meet daily requirements, 2.5 % minerals and 3 % moisture.

25

13 g of this powder is mixed in 100 ml of water. The obtained composition is an infant formula particularly intended for at risk infant to cow's milk allergy.

**Example 8: tolerogenic infant formula**

5 In order to obtain a tolerogenic infant formula, we prepare the following mixture containing for 100 ml of formula, 1.6 % peptides (tolerogenic peptides as prepared in example 2, represent about 0.1 to 0.2 % of total peptides), 3.4 % fat, 7.4 % carbohydrates (including lactose 5.2 %, maltodextrin 1.4 %, starch 0.8 %), traces of vitamins and oligoelements to meet daily requirements, 0.3 % of minerals and 79.9 % of water.

10 **Exemple 9:**

15 In order to obtain a tolerogenic preparation of a small volume, we prepare the following mixture containing for 30ml of preparation, from about 0.003% to about 0.015% of tolerogenic peptides (tolerogenic peptides as prepared in example 2, represent 100% of total peptides), 7.4% of carbohydrates (including maltodextrin 1.4% and saccharose 6%), 92.5% of water and aroma. In order to induce oral tolerance, this preparation is to be administered 1 to 5 times daily , to newborns « at risk » of cow's milk allergy either fed hypoallergenic formula or breastfed.

20

## Claims

1. A hypoallergenic composition for the induction of protein tolerance in at risk individuals of protein allergy, containing (i) a "non allergenic" protein material extensively hydrolysed basis and/or of (ii) a free amino acid basis, said composition comprising as the active ingredient at least one tolerogenic peptide of the allergenic protein.
2. A composition according to claim 1, wherein said tolerogenic peptides are present in the form of (i) isolated tolerogenic peptidic fractions of the hydrolysis of proteinaceous material containing the allergenic protein and/or (ii) synthetically prepared tolerogenic peptides.
3. A composition according to any of claims 1 to 2, which contains tolerogenic peptides or selected tolerogenic peptidic fractions of hydrolysis in such an amount that the ratio of tolerogenic activity by residual antigenicity is at least of about  $2 \times 10^{-2}$ .
4. A composition according to any of claims 1 to 3, which contains an amount of tolerogenic peptide of about 0.01 % to 10%, and preferably about 0.1 % to 0.2 % of the total nitrogen source of the proteins in the composition.
5. A composition according to any of claims 1 to 4, intended for mammals susceptible to cow's milk allergy, which contains a peptidic fraction comprising  $\text{H}_2\text{N-I-D-A-L-N-E-N-K-COOH}$ ,  $\text{H}_2\text{N-V-L-V-L-D-T-D-Y-K}_2\text{-K-COOH}$  or  $\text{H}_2\text{N-T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH}$  from  $\beta$ -lactoglobulin.
6. A composition according to any of claims 1 to 5, which contains a source of nitrogen providing 7 to 25% of the total energy, a source of carbohydrates which provides 28 to 66% of the total energy, a source of lipids which provides 25 to 60% of the total energy, minerals and vitamins to meet daily requirements and at least one of said tolerogenic peptides that is added in an efficient quantity so as to induce oral tolerance.
7. The use of tolerogenic peptide of milk protein selected for its ability to induce oral tolerance to milk proteins, for the preparation of a hypoallergenic composition intended for mammals susceptible to cow's milk allergy.

- 5
8. The use according to claim 6 wherein tolerogenic peptide is from milk origin and particularly from  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, bovin serum albumin or casein origin.
9. The use according to any of claims 6 to 7, wherein tolerogenic peptide comprises  $\text{H}_2\text{N-I-D-A-L-N-E-N-K-COOH}$ ,  $\text{H}_2\text{N-V-L-V-L-D-T-D-Y-K}_2\text{-K-COOH}$  or  $\text{H}_2\text{N-T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH}$  from  $\beta$ -lactoglobulin.
- 10
10. The use of tolerogenic peptide according to any of claims 6 to 8 in an amount of about at least 0.01% to 10% (Nitrogen source of the protein).
11. A method of preparation of tolerogenic peptide useful in the induction of protein tolerance in at risk individuals to protein allergy, wherein :
- 15
- (i) a proteinaceous material containing the allergenic protein is hydrolysed to a degree of hydrolysis of about 10 to 50 %;
- (ii) then treated to inactivate residual enzyme activity;
- (iii) the protein hydrolysate solution is clarified and submitted to precipitation treatment or passed into a chromatography column filled
- 20
- with appropriated resin to extract tolerogenic peptidic fractions
12. A method according to claim 11, wherein tolerogenic peptidic fractions are concentrated to a dry solids content of about 35-65 % and spray dried.
- 25

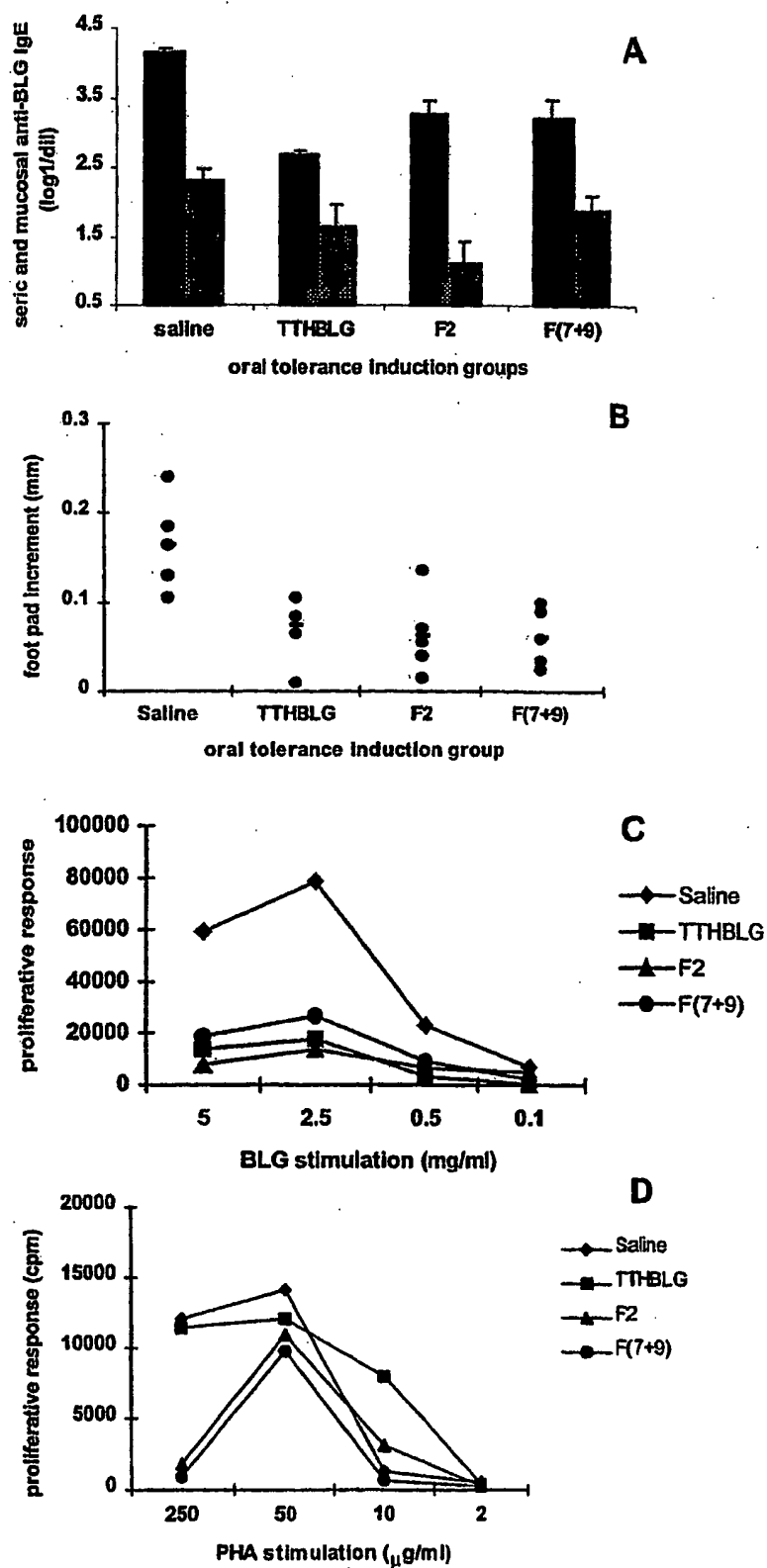


Figure 1



Peptide T1 (139-141)	H <sub>3</sub> N-A-L-K-COOH	Peptide T2 (146-148)	H <sub>3</sub> N-H-I-R-COOH	Peptide T3 (136-138)	H <sub>3</sub> N-F-D-K-COOH
Peptide T4 (71-75)	H <sub>3</sub> N-I-I-A-E-K-COOH	Peptide T5 (62-69)	H <sub>3</sub> N-E-N-G-E-C-A-Q-K-COOH		
Peptide T6 (84-91)	H <sub>3</sub> N-I-D-A-L-N-E-N-K-COOH	Peptide T7 (9-14)	H <sub>3</sub> N-G-L-D-I-Q-K-COOH		
Peptide T8 (142-145)	H <sub>3</sub> N-A-L-P-M-COOH	Peptide T9, T10, T11 (61-67, -68, -69)	H <sub>3</sub> N-W-E-N-G-E-C-A, -Q, -K, -COOH		
Peptide T12 (125-135)	H <sub>3</sub> N-T-P-E-V-D-D-E-A-L-E-K-COOH	Peptide T13 (1-8)	H <sub>3</sub> N-L-I-V-T-Q-T-M-K-COOH		
Peptide T14 (142-148) = T8 (142-145) + T2 (146-148)	H <sub>3</sub> N-A-L-P-M-H-I-R-COOH	Peptide T15 (78-82)	H <sub>3</sub> N-I-P-A-V-F-K-COOH		
Peptide T16 (15-20)	H <sub>3</sub> N-V-A-G-T-W-Y-COOH	Peptide T17 (92-99, 100)	H <sub>3</sub> N-V-L-V-L-D-T-D-Y-K, -K-COOH		
Peptide T18 (125-138) = T12 (125-135) + T3 (136-138)	H <sub>3</sub> N-T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH				
Peptide T19 (25-40)	H <sub>3</sub> N-A-A-S-D-I-S-L-L-D-A-Q-S-A-P-L-R-COOH				
Peptide T20, T21 (61, 62-69):S-S:(149-162)		H <sub>3</sub> N-W, -E-N-G-E-C-A-Q-K-COOH			
		H <sub>3</sub> N-L-S-F-N-P-T-Q-L-E-E-Q-C-H-I-COOH			
Peptide T22 (21-40)	H <sub>3</sub> N-S-L-A-M-A-A-S-D-I-S-L-L-D-A-Q-S-A-P-L-R-COOH				
Peptide T23 (41-60)	H <sub>3</sub> N-V-Y-V-E-E-L-K-P-T-P-E-G-D-L-E-I-L-L-Q-K-COOH				
Peptide T24 (149-162)	H <sub>3</sub> N-L-S-F-N-P-T-Q-L-E-E-Q-C-H-I-COOH				

Figure 2

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 00/00334

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC 7 A23J3/34 A23L1/305 A61K38/01		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
IPC 7 A23J A23L A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
WPI Data, FSTA, EPO-Internal, CHEM ABS Data, EMBASE		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	LO C.W. AND KLEINMAN R.E.: "Infant formula, past and future: opportunities for improvement" AMERICAN JOURNAL OF CLINICAL NUTRITION, vol. 63, no. 4, 1996, page 646S-650S XP002102415	1-10
X	abstract	11,12
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<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
14 June 2000		26/06/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer  De Jong, E

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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